AD_____

Award Number: DAMD17-03-1-0534

TITLE: Targetable Endolytic Protein-based Polymers for Systemic Breast Cancer Gene Therapy

PRINCIPAL INVESTIGATOR: Hamid Ghandehari, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland at Baltimore
Baltimore, MD 21201

REPORT DATE: August 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-08-05	Final	07/07/03-07/06/05

4. TITLE AND SUBTITLE
Targetable Endolytic Protein-based Polymers for Systemic Breast Cancer Gene Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0534

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hamid Ghandehari, Ph.D.

Email-hghandeh@rx.umaryland.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland at Baltimore
Baltimore, MD 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

Abstract can be found on next page.

15. SUBJECT TERMS
gene therapy, targeted therapies, systematic therapies, discovery and development, localized therapies, drug delivery

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	62	19b. TELEPHONE NUMBER *(include area code)* 301-619-7325

ABSTRACT

Using recombinant techniques the first prototype polymer-protein fusion was biosynthesized to mediate transgene expression in mammalian cell lines. The carrier was designed to have the structure of $(KHKHKHKHKK)_6$-FGF2 where Lysine (K) residues would allow complexation with plasmid DNA by electrostatic interactions, basic Fibroblast Growth Factor (FGF2) to target cells over-expressing FGF2 receptors (FGFR), and Histidine (H) residues to facilitate escape from the endosomoal compartments due to proton sponge effect. The gene carrier was biosynthesized in *E.coli*, purified, characterized, complexed with pDNA (pEGFP) and the complexes were used to transfect mammalian cells. Results demonstrate the successful cloning, expression, purification and bioactivity of the carrier. The carrier / DNA complexes transfected mammalian cells known to express FGFR. Genetic engineering techniques show promise for systematic investigation of structure-activity relationship of non-viral polymeric vectors.

Table of Contents

Introduction

The **overall purpose** of this project was to develop targetable polymers for systemic breast cancer gene therapy. **The rationale** is that by controlling polymer structure using genetic engineering techniques, it is possible to establish a correlation between structure and transfection efficiency. Three Specific Aims were proposed:

1) To synthesize and characterize copolymers with the general structures of $(KHKHKHKHKK)_n$-(FGF2) using genetic engineering techniques.

2) To evaluate the physicochemical characteristics of complexes of the copolymers with a model reporter plasmid DNA.

3) To evaluate the transfection efficiency of the copolymers in breast cancer cell lines over-expressing FGF2 receptors.

All the specific aims were accomplished at the conclusion of the second year (no cost extension period). Results are briefly described below and have been submitted for publication (please see Appendix A). For more details of experiments accomplished in year 1 please see Year 1 report and appendices.

Body

A) Biosynthesis of the proposed polymers:

For months 1-8 the following task and subtasks were proposed. However, the tasks were fulfilled in 15 months.

Task 1. Synthesis of the copolymers

a. Design and synthesis of oligonucleotides encoding the polymers
b. Synthesis of monomer gene segments
c. Synthesis of multimer gene segments
d. Small scale expression and analysis of the polymers

We successfully synthesized oligonucleotides encoding $(KHKHKHKHKK)_2$ followed by multimerization rendering the $(KHKHKHKHKK)_6$ gene or from this point forward referred to $(KH)_6$. The $(KH)_6$ gene was cloned along with FGF2 gene in pET21b expression vector using genetic engineering techniques and expressed. The expression step was the limiting factor since charged repeating sequences tend to be rigid and unstable during the expression and purification process and tend to be degraded to smaller fragments. The expressed $(KH)_6$-FGF2 was purified (>95% purity) and its expression verified using western blot analysis, MALDI-TOF and amino acid content analysis.

B) Complexation with DNA and characterization of complexes:

For months 9-10 the following task and subtasks were proposed:

Task 2. Complexation with DNA and characterization of complexes

a. Gel retardation assays
b. Zeta potential measurements
c. Size measurements

Complexation and characterization of the complexes for the new polymers were fulfilled in months 16-17 since small quantities of polymers first needed to be expressed and purified. $(KH)_6$-FGF2 was complexed with pDNA (pEGFP) and condensed into nano-size particles. The size of the particles were measured by photon correlation spectroscopy and determined to be 231±15 nm. Task b was found extremely difficult to accomplish as the construct is a protein which is stable only in buffered solutions with the presence of at least 20mM NaCl. For zeta potential measurements, the solution needs to be salt free. Therefore, this test was excluded from the list.

C) Complexation of vector with pEGFP and transfecting NIH 3T3, T-47D and COS-1 mammalian cell types.

Task 3. Evaluation of the transfection efficiency of the copolymers in breast cancer cell lines over-expressing FGF2 receptors.

The copolymers were complexed with pEGFP and used to transfect non-cancer and cancer cell lines. The complexes successfully transfected NIH3T3 (mouse embryo fibroblast), COS-1 cells (African green monkey kidney), and T47-D cells (human breast cancer) known to over-express FGF2 receptors. This task was done in months 18-21.

Key Research Accomplishments

a) Cloning of the lysine-histidine monomer gene
b) Preparation of DNA encoding lysine-histidine concatamers
c) Preparation of the pAAG vector
d) Cloning of the concatamer DNA
e) Amplification of gene encoding FGF2
f) Amplification of gene encoding poly lysine-histidine
g) Cloning of FGF2 gene into pET21b expression vector
h) Cloning of FGF2 and poly lysine-histidine as a fused gene into pET21b expression vector
i) Expression of the $(KH)_6$-FGF2 copolymer
j) Complexation of $(KH)_6$-FGF2 with pEGFP and characterization
k) Determination of mitogenic activity and toxicity of $(KH)_6$-FGF2
l) Evaluating the transfection efficiency of $(KH)_6$-FGF2 in NIH 3T3, T-47D and COS-1 mammalian cell types

Reportable Outcome

A) Manuscripts:

H. Hatefi, Z. Megeed, and H. Ghandehari, Recombinant Polymer-Protein Fusion: A Promising Approach towards Efficient and Targeted Gene Delivery, *Nature Materials,* submitted. *(This is an original research manuscript that summarizes research accomplished for this grant and can serve also as a technical report addendum, Please see Appendix A).*

M. Haider, H. Hatefi, and H. Ghandehari, Recombinant Polymers for Cancer Gene Therapy: a Minireview, *Journal of Controlled Release,* submitted. *(This is a review article. Part of the work pertaining to this grant (biosynthesis) is reviewed in this article - please see Appendix B).*

B) Presentations:

The following abstracts included all or part of the work related to this grant application:

Z. Megeed, J. Cappello, D. Li, B. O'Malley, and H. Ghandehari, Genetically Engineered Biomaterials for Viral & Nonviral Cancer Gene Therapy, 31st International Symposium on Controlled Release of Bioactive Materials, Honolulu, Hawaii, June 13-16, 2004. Proceedings. Podium presentation.

H. Ghandehari, M. Haider, R. Dandu, and A. Hatefi, Protein-Based Polymers for Cancer Gene Therapy, Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Baltimore, MD, November 7-11, 2004. Proceedings. Invited talk.

A. Hatefi and H. Ghandehari, Targetable and Endolytic Recombinant Copolymer for Systemic Gene Delivery: Biosynthesis, 12th International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, Utah, February 21-24, 2005. Proceedings. Poster presentation.

H. Ghandehari, M. Haider, J. Cappello, R. Dandu, and A. Hatefi, Recombinant Polymers for Cancer Gene Therapy, 12th International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, Utah, February 21-24, 2005. Proceedings. Invited talk.

A. Hatefi, and H. Ghandehari, Design and Biosynthesis of a Genetically Engineered Non-Viral Vector for Breast Cancer Gene Therapy, 32nd International Symposium on Controlled Release of Bioactive Materials, Miami, Florida, June 18-22, 2005. Proceedings. Poster presentation.

B2. Invited talks

As *part of* the effort in our lab related to recombinant polymers for cancer gene therapy, progress made for this project was included in invited talks given by the PI in the following venues (followed by title of talk in italic):

International Symposium on Controlled Release of Bioactive Materials, Honolulu, Hawaii, June 12, 2004. *Controlled Drug and Gene Delivery from Genetically Engineered Polymers* presented in Workshop on Genetically Engineered Polymers for Drug Delivery and Biomedical Applications (also organizer of the workshop).

Symposium on Nanomedicine and Drug Delivery, Polytechnic University, Brooklyn, NY, August 19, 2004. *Recombinant Polymers as Constructs for Nanomedicine.*

Annual Meeting of the American Association of Pharmaceutical Scientists, Baltimore, MD, November 10, 2004. *Protein-Based Polymers for Cancer Gene Therapy.*

International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, Utah, February 21-24, 2005. *Recombinant Polymers for Cancer Gene Therapy.*

University of Kentucky, Department of Pharmaceutical Sciences, Lexington, Kentucky, March 11, 2005. *Engineering Polymers for Targeted Drug and Gene Delivery.*

Medtronic, Inc. Minneapolis, Minnesota, May 27, 2005. *Engineering Polymers for Targeted Delivery of Bioactive Agents.*

International Symposium on Controlled Release of Bioactive Materials, Miami, Hawaii, June 18, 2005. *Genetic Engineering of Polymers for Controlled Release.*

C) Patents:

A provisional patent has been filed:

Hamid Ghandehari, Arash Hatefi, Zaki Megeed; Recombinant Polymers For Systemic Gene Delivery: Provisional Patent - Dockett Number UMB-001-009P-filed February 17, 2005.

D) Training and employment opportunities supported based on this award:

A small portion of one of my former students' dissertation involved parts of this project. *Zak Megeed* obtained his PhD in Fall of 2003. Currently he is a postdoctoral fellow at Harvard Center for Engineering and Medicine with a long-term goal of pursuing a courier in academia. He worked on the cloning of the KH multimer gene segments.

Subsequently postdoctoral fellow *Dr. Arash Hatefi* pursued this research in our laboratory. He cloned and expressed the polymer, complexed it with plasmid DNA and

transfected the cells. He plans to pursue an academic courier in pharmaceutical sciences and drug delivery.

E) Funding applied based on this award

Dr. Hatefi, under the supervision of the PI, applied for a postdoctoral fellowship from the National Cancer Center based on the accomplishments of this work which was funded. It supplemented the continuation of this work. Preliminary data obtained from this Concept Award are included as supportive contents for two pending grants (PI: Ghandehari), one NIH and concurrent submission to DOD, focused on breast cancer gene therapy.

Conclusions

In summary progress was made in all of the specific aims with some pitfalls in the expression and purification step leading to the no cost extension. The first prototype genetically engineered polymer, $(KH)_6$-FGF2 was biosynthesized. This polymer is able to mediate gene transfer in various cell lines and constitutes an encouraging starting point for the biosynthesis of recombinant gene carriers with repetitive sequences for studying structure-transfection efficiency relationships. This work provides a platform for the subsequent biosynthesis of additional analogues where the ratio of Lys:His, the length of Lys-His repeats and the location of targeting moiety among other factors can be systematically changed by recombinant techniques. By doing so, one can gain insight into the effect of these detailed structural changes on transfection efficiency, cytotoxicity and ultimately preclinical and clinical use in human cancer gene therapy.

References

None

Appendices:

Appendix A: H. Hatefi, Z. Megeed, and H. Ghandehari, Recombinant Polymer-Protein Fusion: A Promising Approach towards Efficient and Targeted Gene Delivery, *Nature Materials,* submitted. *(This is an original research manuscript that summarizes research accomplished for this grant and can serve also as a technical report addendum).*

Appendix B: M. Haider, H. Hatefi, and H. Ghandehari, Recombinant Polymers for Cancer Gene Therapy: a Minireview, *Journal of Controlled Release,* submitted. *(This is a review article. Part of the work pertaining to this grant (biosynthesis) is reviewed in this article).*

Recombinant Polymer-Protein Fusion: A Promising Approach towards Efficient and Targeted Gene Delivery

Arash Hatefi, Zaki Megeed*, and Hamidreza Ghandehari

Department of Pharmaceutical Sciences and Center for Nanomedicine and Cellular Delivery, University of Maryland, Baltimore, Baltimore, Maryland, USA 21201

Correspondence should be addressed to:

Hamidreza Ghandehari, Ph.D

hghandeh@rx.umaryland.edu

Centre for Nanomedicine and Cellular Delivery

University of Maryland School of Pharmacy

Department of Pharmaceutical Sciences

20 Penn Street, HSFII Room 625

Baltimore, Maryland 21201-1075

Phone: (410) 706-8650

Fax: (410) 706-5017

*Present Affiliation: The Center for Engineering in Medicine, Massachusetts General Hospital, and Harvard Medical School

Appendix A: H. Hatefi, Z. Megeed, and H. Ghandehari, Recombinant Polymer-Protein Fusion: A Promising Approach towards Efficient and Targeted Gene Delivery, *Nature Materials,* submitted-July 2005. *(This is an original research manuscript that summarizes research accomplished for this grant and can serve also as a technical report addendum).*

Keywords: / Recombinant Polymers/ Targeted Delivery/ Gene delivery/ Cancer

Abstract

Using recombinant techniques the first prototype polymer-protein fusion was biosynthesized to mediate transgene expression in mammalian cell lines. The carrier was designed to have the structure of $(KHKHKHKHKK)_6$-FGF2 where Lysine (K) residues would allow complexation with plasmid DNA, basic Fibroblast Growth Factor (FGF2) to target cells over-expressing FGF2 receptors (FGFR), and Histidine (H) residues to facilitate escape from the endosomoal compartments. The protein polymer carrier was biosynthesized in *E.coli*, purified, characterized, complexed with pDNA and the complexes were used to transfect NIH 3T3, T47-D and COS-1 mammalian cell types known to express FGFR. Results demonstrate the successful cloning, expression, purification and bioactivity of the carrier. Genetic engineering techniques show promise for systematic investigation of structure-activity relationship of non-viral polymeric vectors.

Introduction

A critical challenge in the realization of gene therapies is the lack of safe and clinically effective vectors that can be administered to target a specific site of action[1]. For systemically administered vectors to be maximally effective, they should: a) protect the nucleic acid from degradation in the blood stream, b) be specifically recognized and internalized by target cells, c) promote escape from the endosomal compartment, and d) direct the nucleic acid towards the desired site of action, most frequently the nucleus[2, 3]. Viruses have evolved the means to efficiently overcome these barriers, but their broad application has been limited by safety issues. Synthetic (non-viral) polymeric vectors have the potential to circumvent virus-associated safety issues, but their transfection efficiency generally remains orders of magnitude lower than that mediated by viruses[4, 5]. A major factor limiting the design of polymeric carriers with improved activity is the limited understanding of the relationship between polymer structure and transfection efficiency. To study the critical steps involved in an efficient transgene expression, there is a need for novel approaches that allow a systematic correlation between polymer structure and properties necessary for successful gene transfer. Polymeric vectors are generally synthesized by traditional chemical methods, which result in the production of polymers with random sequences (for co-polymers), polydispersity, and difficulty in attaching functional motifs at precise locations. As a result, the establishment of systematic polymer structure - activity relationships is usually confounded by the random nature of these materials.

Recombinant DNA technology has enabled the production of large molecular weight polypeptides (*protein polymers*) containing repeating blocks of amino acids with precise compositions, sequences and lengths[6-8]. We have hypothesized that artificial protein polymers can be combined with naturally occurring protein domains to introduce

functionality and generate simple or complex gene delivery vectors with predictable and controlled properties[9]. This approach (the insertion of one protein into another), also known as domain insertion, is a strategy previously used to create molecular switches[10], voltage-gated channels and molecular sensors[11]. Previously, we examined the potential of recombinant polymers for controlled release and matrix-mediated *localized* gene delivery[12-15]. Given the high degree of control over polymer structure the idea is that protein polymers can enable a systematic structure-function relationship for gene delivery applications. The merits of this technology however have not been investigated for *systemic* gene delivery. Recombinant polymers can be designed to incorporate motifs to condense and protect DNA (e.g., cationic residues)[16], target specific cell types upon systemic administration (e.g., receptor ligands)[17, 18], disrupt endosomes (e.g., fusogenic peptides)[19-21], and traffic cargo to specific sub-cellular compartments (e.g., nuclear localization signals)[22]. By doing so it is possible to strategically correlate polymer structure with function with the aim of increasing transfection efficiency and safety. In addition, advantages such as cost-effective large-scale manufacturing, purity, homogeneity, and biocompatibility make recombinant polymers attractive alternatives to conventional non-viral gene vectors.

In this study, we present the biosynthesis and *in-vitro* characterization of the first prototype recombinant polymer for targeted gene delivery. The protein polymer with the structure $(KHKHKHKHKK)_6$-FGF2 was designed (from this point forward referred to $(KH)_6$-FGF2). It contains 36 lysine residues (K) in the $(KH)_6$ segment, which condense DNA, and 24 histidine residues (H) to promote endosomal escape via the proton sponge hypothesis[23, 24]. At the C-terminus of the construct, FGF2 represents fibroblast growth factor 2, a ligand for the basic fibroblast growth factor receptor (FGFR), known to be over-expressed in a sub-population of breast cancer cells, thus conferring the potential for targeted delivery by receptor-mediated endocytosis[25]. As a starting point, the sequence of

the KH tail was chosen arbitrarily, while keeping the lysine to histidine ratio constant at 6:4. This ratio was chosen based on previous studies conducted by Midoux and Monsigny (1999)[26].

Results

$(KH)_6$-FGF2 Cloning, Expression and Identification

Using the cloning strategy shown in **Fig. 1**, the $(KH)_6$-FGF2 protein polymer was cloned and expressed and the fidelity of the construct was confirmed by DNA sequencing, yielding the following amino acid sequence (KH repeats are in bold and FGF2 sequence is underlined): M**KHKHKHKHKKKHKHKHKHKKHKHKHKHKKKHKHKHKHKK HKHKHKHKKKHKHKHKHKK**EDPAAEFMAAGSITTLPALPEDGGSGAFPPGHFK DPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKGVCANRY LAMKEDGRLLASKCVTDECFFFERLESNNYNTYRSRKYTSWYVALKRTGQYKLG SKTGPGQKAILFLPMSAKSKLAAALEHHHHHH.. The purity and expression of the protein was determined by SDS-PAGE **(Fig. 2A)** and western blot analysis using rabbit Anti-FGF2 (data not shown) and mouse Anti-6XHis **(Fig. 2B)**. The molecular weight of $(KH)_6$-FGF2 was determined by MALDI-TOF to be 27,402. The results of the amino acid content analysis agreed with the expected amino acid compositions.

Plasmid DNA Condensation and Particle Size Analysis

The ability of the $(KH)_6$-FGF2 vector to condense model plasmid DNA (pEGFP) was examined in the presence of fetal bovine serum 10% (v/v). Gel retardation assays indicate that the vector does interact with DNA, retarding its migration and accessibility to ethidium bromide in a dose-dependent manner **(Fig. 3)**. The size of the pDNA/vector complexes was determined to be 231 ± 15 nm by photon correlation spectroscopy.

Mitogenic Activity and Toxicity of $(KH)_6$-FGF2

The bioactivity of the FGF2 segment of $(KH)_6$-FGF2 was evaluated with WST-1 cell proliferation assay in NIH 3T3 fibroblasts, known to express the FGFR. The FGF2 motif present in $(KH)_6$-FGF2 was shown to be active in terms of inducing cell proliferation in fibroblasts when they were exposed to concentrations of vector that mimicked physiological FGF2 levels **(Fig. 4)**. The toxicity of $(KH)_6$-FGF2 in NIH 3T3 cells (grown in serum free media or complete growth media) exposed to super-physiological doses of vector ranging from 10 to 30,000 $ng.ml^{-1}$ was also determined. It was observed that $(KH)_6$-FGF2 did not have any deleterious effect on the cell proliferation rate regardless of the dose or growth media.

In-vitro Cell Transfection Mediated by $(KH)_6$-FGF2

To evaluate transfection efficiency, the pEGFP plasmid, encoding green fluorescent protein (GFP) was condensed with $(KH)_6$-FGF2 and used as a reporter to monitor the percentage of transfected cells in three cell types expressing FGFR: NIH 3T3, COS-1, and T47-D cells, in the presence and absence of serum. Transfection was observed in all cell lines, regardless of whether serum was present, though the percentage of transfected cells was significantly higher in the absence of serum. Transfection efficiencies ranging from 15 to 41 % were observed in the absence of serum and 4 to 10 % in the presence of serum **(Fig. 5)**. To evaluate whether specific uptake was occurring through FGFR, we conducted transfection experiments on NIH 3T3 cells in the presence of 1000 $ng.ml^{-1}$ free FGF2. Under these conditions, we observed a reduction in transfection efficiency of approximately 85% (from 28±9 % to 4±2 %).

Discussion

Previous work demonstrated that the peptides comprised of lysine and histidine can form a complex and partially neutralize the negative charge of the plasmid DNA, and the histidine component with a pKa of 6.0, buffers and aids in the release of plasmid DNA from endosomal vesicles[19, 24, 26]. In addition, FGF2 had been attached to polylysine via a non-specific disulfide bond to target cells overexpressing FGFR[18]. While these constructs show promise in gene transfer, given the inherent limitations in the synthetic strategy for peptides or polymeric carriers, it is difficult to systematically correlate structure with function. As a first step towards that goal, we genetically engineered a prototype construct containing all these motifs in one polymer chain.

The molecular weight of $(KH)_6$-FGF2 was determined by MALDI-TOF to be 27,402 which was very close to the theoretical value of 27,313 daltons, the slight difference potentially relating to the peak width at half height calculations by the instrument. Amino acid content analysis also provided more information regarding the identity of the expressed protein which showed good agreement between the expected and observed percentage of each amino acid in the structure of the protein. These results demonstrated the successful biosynthesis of the $(KH)_6$-FGF2 vector. We then evaluated the ability of the vector to act as a functional gene carrier.

Cationic amino acids, such as lysine, condense DNA by neutralizing the negative charges of phosphate groups and, therefore, decreasing the columbic repulsions between DNA phosphates and promoting hydrophobic interactions at the complexed sites[27]. Also, the stability of condensed DNA in physiological conditions is one of the major hurdles for its use in gene therapy. Even though DNA particles are shown to be stable in salt-free environments, in the presence of physiological saline or serum they often fail to remain stable[27]. **Fig. 3** clearly shows the vulnerability of naked pDNA to endonucleases present in serum (Lane 1 and 2). By adding $(KH)_6$-FGF2 to pDNA in escalating concentrations, the

negative charges on pDNA were neutralized and reduced electrophoretic mobility was observed. It was also demonstrated that $(KH)_6$-FGF2 can neutralize the charge on pDNA (pEGFP) in 1:100 mole/mole ratio **(Fig. 3, Lane 6)** and remained complexed in the presence of serum proteins and salts. pEGFP has 4731 base pairs which corresponds to 9462 negative charges while (KH)6-FGF2 has 60 (49 lys + 11 Arg) basic residues which corresponds to 60 positive charges. Considering the full condensation of 1 mole pEGFP with 100 mole $(KH)_6$-FGF2 as determined by gel retardation assay, every 1.6 negative charges (N) was neutralized by 1 positive charge (P). Condensation of every 1.6 negative charges with 1 positive charge is perhaps due to the active contribution of histidine residues in DNA condensation via hydrogen bond formation[28, 29]. Considering the total number of histidine and lysine residues in DNA condensation calculations, the N/P ratio was determined to be 1:1. Therefore, it is plausible that not only cationic residues are important in DNA condensation, residues such as histidine which can form a hydrogen bond with DNA may also play a role.

Beside DNA complexation, the size of the complexes also plays an important role in DNA internalization. Although there is no consensus on whether particles with smaller size are more effective than larger ones, complexes should at least be small enough to be endocytosed. The average particle size of the vector - DNA complexes, at a ratio of 1/100, was 231 ± 15 nm.

FGF2 is a growth factor that induces proliferation in cells expressing FGFR[30]. In this case, the ability of the FGF2 domain to bind FGFR is critical to our goal of targeting cells over-expressing FGFR. Proteins are known to degrade rapidly or lose their activity when their conformations are altered by mutations, incorporation of amino acid analogs, denaturation or premature chain terminations[31]. These modifications may prevent proper folding or disrupt protein structure, which can make the resulting aberrant protein prone to

degradation or inactive. This is especially important in fusion proteins, where one domain may interfere with another and render it inactive. $(KH)_6$-FGF2 is a fusion polymer-protein comprised of two segments, $(KH)_6$ and FGF2 in which one may affect the other, resulting a vector that lacks the ability to condense DNA or bind FGFR. To test the FGF2 activity, NIH 3T3 cells, which express FGFR, were treated with $(KH)_6$-FGF2 concentrations close to FGF2 concentrations in the physiologic range (i.e., 0.1-10 $ng.ml^{-1}$). Results indicate that $(KH)_6$-FGF2 significantly enhanced cell proliferation, indicating that the addition of a lysine-histidine tail to the FGF2 did not inactivate the FGF2 domain.

The toxicity of $(KH)_6$-FGF2 was also examined. It has been reported that high molecular weight cationic polymers such as poly-lysine are moderately toxic in mammalian cell culture[32]. The toxicity of $(KH)_6$-FGF2 was studied under two conditions; a) in a serum free media where no protein but transferrin and insulin are present; and b) in a complete growth media supplemented with serum. Since the transfection studies were performed under both serum-free and serum-containing conditions, this study provided an insight whether the vector had any toxic effect on cells at high concentrations, which could negatively impact transfection efficiency. It was observed that $(KH)_6$-FGF2 did not have any deleterious effect up to 30 $\mu g.ml^{-1}$ on the cell proliferation rate regardless of the dose or growth media.

Having shown that the $(KH)_6$-FGF2 is biologically active and can bind pDNA, we further examined the ability of the vector to deliver pDNA into cells expressing FGFR. NIH 3T3, COS-1 and T-47D are known to express FGFR[18, 33]. Cell transfection was conducted in serum free media (SFM) and growth media supplemented with serum (complete growth media). In complete growth media proteins are present which might interact with $(KH)_6$-FGF2/pEGFP complexes and the presence of growth factors in serum can inhibit the receptor mediated endocytosis of complexes. In contrast, SFM does not have

any growth factors that compete with $(KH)_6$-FGF2 and there are only two proteins (i.e., insulin and transferrin) present in the media, which reduces the possibility of interaction with complexes. NIH 3T3, T47-D and COS-1 cells were transfected under both conditions. As expected, a higher percentage of cells were transfected in SFM in comparison with cells transfected in the presence of complete growth media **(Fig. 6)**. Serum proteins and growth factors may have contributed to the reduction in transfection efficiency observed in complete medium.

To determine whether $(KH)_6$-FGF2 complexed with pEGFP is specifically delivered to cells by FGF2 receptor-mediated endocytosis, we performed an inhibition study in SFM in the presence of 1000 $ng.ml^{-1}$ free FGF2 as a competitor. Under these conditions **(Fig. 5)**, we observed a reduction in transfection efficiency of approximately 85% (from 28±9 % to 4±2 %). The lack of full inhibition could be the result of particle uptake via non-specific endocytosis.

It can be concluded that the $(KH)_6$-FGF2 vector is able to mediate gene transfer in various cell lines and constitutes an encouraging starting point for the biosynthesis of recombinant protein polymers with repetitive sequences for studying structure-transfection efficiency relationships. This work provides a platform for the subsequent biosynthesis of additional analogues where the ratio of Lys:His, the length of Lys-His repeats and the location of targeting moiety among other factors can be systematically changed by recombinant techniques. By doing so, one can gain insight into the effect of these detailed structural changes on transfection efficiency, cytotoxicity and ultimately preclinical and clinical use in human cancer gene therapy.

Materials and Methods

Cloning of the KH concatemers

Oligonucleotides encoding $(KHKHKHKHKK)_2$ were designed to maximize the use of preferred codons in *E. coli*, while minimizing the codon repetition of the monomer gene. Oligonucleotides encoding the monomer with *BamH I* and *EcoR I* (Shown in Bold), 5'-AGTTA**GGATCC**CTCTTCAAAGCACAAACATAAGCACAAGCACAAGAAGAAAC ATAAACACAAGCATAAACACAAAAAGTGAAGAG**GAATTC**TAACT-3', were annealed, digested with these enzymes, and cloned into the pZero-2 (Invitrogen, CA, USA) cloning vector, using standard cloning techniques. The vector was transformed into *E. coli* TOP10 (Invitrogen, CA, USA), which were subsequently plated on LB agar containing kanamycin (37.5 $\mu g.ml^{-1}$). Concatemers were produced by first performing PCR directly on an *E. coli* TOP10 colony containing the $(KH)_2$ gene, followed by digestion with the *Eam 1104 I* (site underlined) restriction enzyme, acrylamide gel purification, and self-ligation with T4 DNA Ligase. $(KH)_n$ concatemers were then cloned into a modified pET19b+ (pAAG) vector.

Cloning of $(KH)_6$-FGF2

The gene encoding FGF2 (generously provided by Dr. Patricia Dell'Era, Dept. Biomedical Sciences & Biotechnology, Unit of General Pathology and Immunology, Brescia, Italy) was amplified by PCR using the following primers: *Forward* (*NdeI* and *EcoRI* sites underlined) GTTCCACATATGGGGGAATTCATGGCAGCCGGGAGCATC A; *Reverse* (*HindIII* underlined): CGGGAAAAGCTTGCTCTTAGCAGACATTGG. The amplified gene was double digested with *NdeI* and *HindIII* and purified by agarose gel electrophoresis. The FGF2 gene was then cloned into a pET21b vector that was previously double digested with *NdeI* and *Hind III.* A gene encoding $(KH)_6$, which corresponds to 30 lysine-histidine repeats (total 60 lysine-histidine) was then amplified from the pAAG

vector by PCR using the primers: *Forward* (*NdeI* site underlined): GACGACGACAAGCATATGAAGCAC; *Reverse* (*EcoRI* site underlined): CGGGTTGAATTCAGCAGCCGGATCCTCCTTTTT. The amplified $(KH)_6$ gene and pET21b-FGF2 were double digested with *NdeI* and *EcoRI* and ligated with T4 DNA ligase to form pET21b-$(KH)_6$-FGF2. This vector was transformed into *E.coli* NovaBlue (Novagen, CA, USA) which was subsequently plated on LB agar containing carbenicillin (100 $\mu g.ml^{-1}$).

Expression and Purification of $(KH)_6$-FGF2

pET21b-$(KH)_6$-FGF2 vector was purified (Qiagen) and transformed into *E.coli* BL21(DE3) (*lon*$^-$, *ompT*$^-$). Transformants were grown at 30°C until the OD_{600} reached 0.7, when recombinant protein expression was induced by the addition of IPTG to a final concentration of 0.2 mM. After 4 hours, cells were harvested by centrifugation, lysed, and centrifuged for 1 hour at 30,000g (4°C) to pellet the insoluble fraction. The soluble fraction containing $(KH)_6$-FGF2 was loaded onto a Ni-NTA column and washed with 20 volumes of wash buffer. The protein was eluted with buffer containing 1M imidazole and analyzed by western blot and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Amino Acid Content Analysis and MALDI-TOF

To determine the amino acid composition and exact molecular weight of the expressed protein, amino acid content analysis and Matrix-Assisted Laser Desorption Ionization Mass Spectroscopy (MALDI-TOF) was performed by Commonwealth Biotechnologies Inc. (Richmond, VA). For amino acid analysis, the sample was hydrolyzed in a gas phase of 6N HCl followed by drying prior to resolubilization and analysis.

Gel Retardation Assay

The formation of pDNA/vector complexes was examined by gel retardation. pDNA (pEGFP, Clontech, Mountain View, CA) was complexed with $(KH)_6$-FGF2 at pDNA:vector molar ratios of 1:40, 1:60, 1:80, and 1:100. In a microfuge tube, 1.2 µg of pDNA was added and diluted to 10 µl with deionized water. In a separate microfuge tube vector was added to produce the desired ratio and diluted to 15 µl with deionized water. The vector solution was added to pDNA solution and incubated at room temperature for 30 minutes. After 30 minutes fetal bovine serum was added to a final concentration of 10% and the complexes were incubated for another hour at 37°C. The complexes were then electrophoresed on a 1% agarose gel and DNA was visualized by ethidium bromide staining.

Cell Proliferation Assay

NIH 3T3 cells were grown in F12/DMEM (1:1 ratio) with 10% fetal calf serum (FCS). At the time of assay, cells were washed with serum-free medium (F12/DMEM supplemented with insulin, transferrin, selenium, fibronectin and dexamethasone) and 5 x 10^3 cells were seeded in a 96 well dish in 150 µl of serum- free media (SFM). A serial dilution of $(KH)_6$-FGF2 was prepared across the plate ranging from 0.02 to 50 $ng.ml^{-1}$. Cells were incubated for 44 hours at 37°C in humidified 5% CO_2 atmosphere. After the incubation time, WST-1 (Roche Applied Science, IN, USA) reagent was added and the absorbance after 4 hours was measured at 440nm. The assay is based on the reduction of WST-1 by viable cells which produces soluble formazan salt. The amount of formazan salt (dye) formed directly correlates to the number of metabolically active cells in the culture medium.

Cell Toxicity Assays

Cell toxicity assays were performed under two conditions: Cells incubated in SFM or in DMEM/F12 supplemented with FCS. In the first case, 5 x 10^3 cells were seeded in a 96 well dish in 150 µl of serum-free media and incubated overnight. A serial dilution of $(KH)_6$-FGF2 was prepared across the plate ranging from 0.01 $\mu g.ml^{-1}$ to 30 $\mu g.ml^{-1}$. The cells were incubated for 4 hours at 37°C in humidified 5% CO_2 atmosphere. After the incubation time, WST-1 reagent was added and the absorbance after 4 hours was measured at 440nm. In the second case, the same study was repeated by replacing SFM with DMEM/F12 90%, FCS 10%. The control cells were treated with Dulbecco's Phosphate Buffer Saline (DPBS) instead of $(KH)_6$-FGF2.

Photon Correlation Spectroscopy

The mean hydrodynamic sizes of plasmid DNA/copolymer complexes were determined by Photon Correlation Spectroscopy (PCS) (Malvern Zetasizer 3000, Malvern Instruments). Measurements were performed in triplicate and reported as mean ± standard error, using an argon laser of 480 nm on complexes formed in water at 25°C and an angle of 90°C. CONTIN analysis was used to fit the experimental intensity decay curve and derive the median particle diameter for the complexes.

Inhibition Study by FGF2

NIH 3T3 cells were seeded in 12 well tissue culture plates at 5 x 10^4 cells per well in 1 ml SFM. Cells were approximately 70-80% confluent at the time of transfection. 5µg/50µl of pEGFP was mixed with 4.2µg/50µl of protein and incubated for 30 minutes at room temperature for complex formation. In one set of wells, FGF2 (1000 $ng.ml^{-1}$) was added

followed by addition of complexes. In the second set, SFM was added followed by addition of complexes (control). The cells were incubated at 37°C in humidified 5% CO_2 atmosphere. After 4 hours, the growth media was removed and replaced with growth media supplemented with serum. Green fluorescent protein activity was visualized using a Zeiss confocal microscope.

Cell Culture and Transfection

NIH 3T3 cells (mouse embryo fibroblast), COS-1 cells (African green monkey kidney), and T47-D cells (human breast cancer) were propagated as suggested by the American Type Culture Collection (VA, USA). Cells were seeded in 12 well tissue culture plates at 4 x 10^4 cells per well in 1 ml growth media (with or without 10% FCS). Cells were approximately 60%-70% confluent at the time of transfection. 5μg/50μl of pEGFP (Green Fluorescent Protein) was mixed with 4.2μg/50μl of protein and incubated for 30 minutes at room temperature for complex formation. The complexes were added to the growth media and the cells were incubated at 37°C in humidified 5% CO_2 atmosphere. After 4 hours, the growth media was removed and replaced with growth media supplemented with serum. Green fluorescent protein activity was visualized using a Zeiss confocal microscope. Lipofectamine 2000 (Invitrogen) complexed with pEGFP was used as a positive control.

Acknowledgements

Financial support was provided by a grant from the Department of Defense Breast Cancer Research Program (DAMD-17-03-1-0534), a Postdoctoral (AH) and a Predoctoral (ZM) Fellowship from the National Cancer Center. We also thank Russel Digate and Zhiyu Li for their helpful discussions.

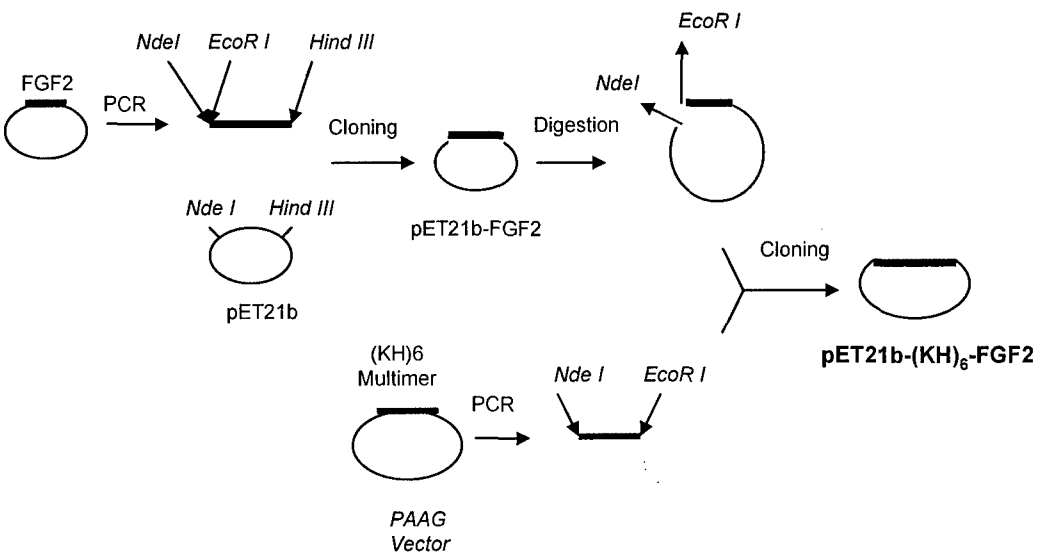


Figure 1. An overview of the cloning strategy used to fuse $(KH)_6$ gene along with FGF2 gene in pET21b expression vector.

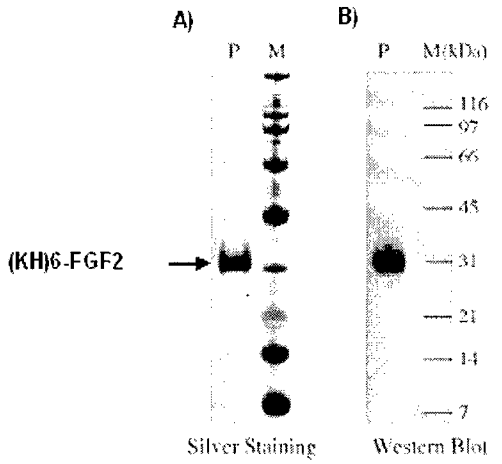


Figure 2: A, SDS-PAGE of purified $(KH)_6$-FGF2; **B,** Western blot analysis of purified $(KH)_6$-FGF2 using Anti 6XHis antibody.

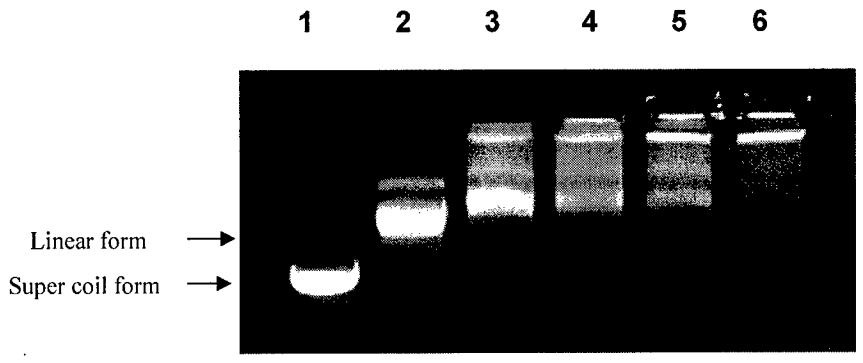


Figure 3. Agarose gel electrophoresis of the DNA/vector complexes. 1) DNA alone, 2) DNA + serum, 3) DNA to vector 1:40 mole/mole, 4) DNA to vector 1:60 mole/mole, 5) DNA to vector 1:80 mole/mole, 6) DNA to vector 1:100 mole/mole.

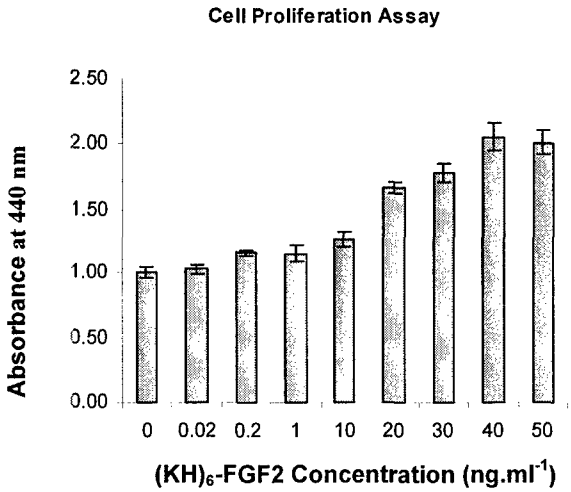


Figure 4. WST-1 cell proliferation assay for NIH 3T3 cells treated with $(KH)_6$-FGF2. Cells were treated with various vector concentrations ranging from 0 (control) to 50 $ng.ml^{-1}$ and the absorbance of soluble formazan was measured at 440nm (Mean± S.D, n=4).

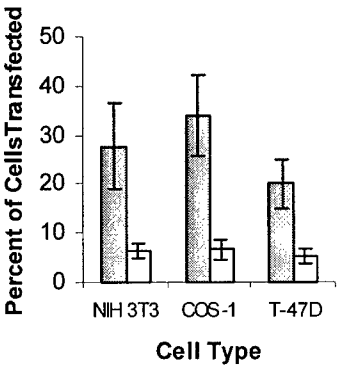
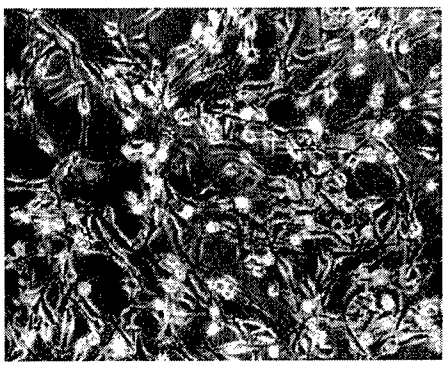


Figure 5. Percentage of cells transfected with $(KH)_6$-FGF2/pEGFP complexes (Mean±S.E, n=9). **Closed bars)** Cells transfected in serum free media. **Open bars)** Cells transfected in growth media containing serum.

a)



b)

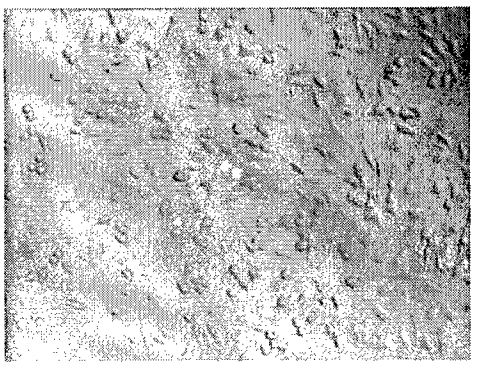


Figure 6: Confocal microscopy image of NIH 3T3 cells transfected with $(KH)_6$-FGF2/pEGFP: : **a)** in serum free media, and **b)** in serum free media with addition of 1000 $ng.ml^{-1}$ FGF2.

References

1. Vile, R. G., Russell, S. J. & Lemoine, N. R. Cancer gene therapy: hard lessons and new courses. Gene Ther 7, 2-8 (2000).
2. Watson, P., Jones, A. T. & Stephens, D. J. Intracellular trafficking pathways and drug delivery: fluorescence imaging of living and fixed cells. Adv Drug Deliver Rev 57, 43-61 (2005).
3. Georgens, C., Weyermann, J. & Zimmer, A. Recombinant virus like particles as drug delivery system. Curr Pharm Biotechnol 6, 49-55 (2005).
4. Lee, E. S., Na, K. & Bae, Y. H. Super pH-sensitive multifunctional polymeric micelle. Nano Lett 5, 325-9 (2005).
5. Mansouri, S. et al. Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy. Eur J Pharm Biopharm 57, 1-8 (2004).
6. Cappello, J. et al. Genetic engineering of structural protein polymers. Biotechnol Prog 6, 198-202 (1990).
7. Krejchi, M. T. et al. Chemical sequence control of beta-sheet assembly in macromolecular crystals of periodic polypeptides. Science 265, 1427-32 (1994).
8. Urry, D. W. et al. in Controlled drug delivery: challenges and strategies (ed. Park K.) 405-438 (American Chemical Society, Washington, DC, 1997).
9. Megeed, Z., & Ghandehari, H. in Polymeric gene delivery: principles and applications (ed. Amiji M.) 489-507 (CRC Press, Boca Raton, FL, USA, 2005).
10. Guntas, G. & Ostermeier, M. Creation of an allosteric enzyme by domain insertion. J Mol Biol 336, 263-73 (2004).
11. Ataka, K. & Pieribone, V. A. A genetically targetable fluorescent probe of channel gating with rapid kinetics. Biophys J 82, 509-16 (2002).
12. Megeed, Z., Cappello, J. & Ghandehari, H. Controlled release of plasmid DNA from a genetically engineered silk-elastinlike hydrogel. Pharm Res 19, 954-9 (2002).
13. Megeed, Z. et al. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. J Control Release 94, 433-45 (2004).
14. Haider, M., Megeed, Z. & Ghandehari, H. Genetically engineered polymers: status and prospects for controlled release. J Control Release 95, 1-26 (2004).
15. Haider, M. et al. Molecular engineering of silk-elastinlike polymers for matrix-mediated gene delivery: biosynthesis and characterization. Mol Pharm 2, 139-50 (2005).
16. Chen, D. J., Majors, B. S., Zelikin, A. & Putnam, D. Structure-function relationships of gene delivery vectors in a limited polycation library. J Control Release 103, 273-83 (2005).
17. Andersson, A., Capala, J. & Carlsson, J. Effects of EGF-dextran-tyrosine-131I conjugates on the clonogenic survival of cultured glioma cells. J Neurooncol 14, 213-23 (1992).
18. Sosnowski, B. A. et al. Targeting DNA to cells with basic fibroblast growth factor (FGF2). J Biol Chem 271, 33647-53 (1996).
19. Leng, Q., & Mixson, A. J. Modified branched peptides with a histidine-rich tail enhance in vitro gene transfection. Nucleic Acids Res 33, e40 (2005).
20. Lee, H., Jeong, J. H. & Park, T. G. A new gene delivery formulation of polyethylenimine/DNA complexes coated with PEG conjugated fusogenic peptide. J Control Release 76, 183-92 (2001).

21. Cho, Y. W., Kim, J. D. & Park, K. Polycation gene delivery systems: escape from endosomes to cytosol. J Pharm Pharmacol 55, 721-34 (2003).
22. Wiseman, J. W., Scott, E. S., Shaw, P. A. & Colledge, W. H. Enhancement of gene delivery to human airway epithelial cells in vitro using a peptide from the polyoma virus protein VP1. J Gene Med (2005).
23. Behr, J. P. The proton sponge: A trick to enter cells the viruses did not exploit. Chimia 51, 34-36 (1997).
24. Putnam, D., Gentry, C. A., Pack, D. W. & Langer, R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc Natl Acad Sci U S A 98, 1200-5 (2001).
25. Blanckaert, V. D. et al. Basic fibroblast growth factor receptors and their prognostic value in human breast cancer. Clin Cancer Res 4, 2939-47 (1998).
26. Midoux, P. & Monsigny, M. Efficient gene transfer by histidylated polylysine/pDNA complexes. Bioconjug Chem 10, 406-11 (1999).
27. Mahato, R. I. Non-viral peptide-based approaches to gene delivery. J Drug Target 7, 249-68 (1999).
28. Zelikin, A. N., Trukhanova, E. S., Putnam, D., Izumrudov, V. A. & Litmanovich, A. A. Competitive reactions in solutions of poly-L-histidine, calf thymus DNA, and synthetic polyanions: determining the binding constants of polyelectrolytes. J Am Chem Soc 125, 13693-9 (2003).
29. Putnam, D., Zelikin, A. N., Izumrudov, V. A. & Langer, R. Polyhistidine-PEG:DNA nanocomposites for gene delivery. Biomaterials 24, 4425-33 (2003).
30. Peyrat, J. P. et al. Basic fibroblast growth factor (bFGF): mitogenic activity and binding sites in human breast cancer. J Steroid Biochem Mol Biol 43, 87-94 (1992).
31. Guruprasad, K., Reddy, B. V. & Pandit, M. W. Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. Protein Eng 4, 155-61 (1990).
32. Brown, M. D. et al. In vitro and in vivo gene transfer with poly(amino acid) vesicles. J Control Release 93, 193-211 (2003).
33. Korah, R. M., Sysounthone, V., Scheff, E. & Wieder, R. Intracellular FGF-2 promotes differentiation in T-47D breast cancer cells. Biochem Biophys Res Commun 277, 255-60 (2000).

Recombinant Polymers for Cancer Gene Therapy: a Minireview

M. Haider[^], A. Hatefi, and H. Ghandehari[*]

Department of Pharmaceutical Sciences, and Center for Nanomedicine and Cellular Delivery, University of Maryland, Baltimore, MD, 21201, USA.

*Corresponding Author:

Hamidreza Ghandehari, Ph.D.
University of Maryland School of Pharmacy
Department of Pharmaceutical Sciences
20 North Penn Street
Baltimore, MD 21201
Tel.: +1-410-706-8650
Fax: +1-410-706-5017
E-mail address: hghandeh@rx.umaryland.edu

Key Words: Cancer gene therapy; Recombinant polymers; Gene delivery; Non-viral vectors; Gene transfer

[^]Current Affiliations: Departments of Biomedical Engineering, The Johns Hopkins University, and Pharmaceutical Sciences, University of Maryland, Baltimore, MD, USA.

Appendix B: Submitted to *Journal of Controlled Release*- June 2005.

Abstract:

A major challenge for successful cancer gene therapy is the development of safe and effective gene delivery vectors. Gene delivery vectors can be viral or nonviral. Among nonviral vectors various polymeric vectors have shown potential in gene delivery. However, much work needs to be done in order to correlate polymer structure with gene release at the target site and transfection efficiency. This article is a brief introduction into cancer gene therapy, barriers and methods for gene transfer with emphasis on the applications of recombinant polymers for cancer gene therapy.

Introduction to cancer gene therapy

Despite advancements and innovations in oncology, cancer continues to be a major cause of suffering and death. The main method of treatment for most solid tumors is still extirpation. It is occasionally combined with radiation and chemotherapy to reduce incidence of tumor recurrence and obtain optimum local disease control. Although radiation appears to have a preferential effect on rapidly dividing cells, it is not specific in its action since it is capable of destroying both normal and malignant cells. Chemotherapeutic agents are cytotoxic drugs used often in combination to obtain a synergistic effect. However, they suffer from significant adverse reactions involving many organ systems.

The fact that cancer originates from a progression of genetic mutations, where one cell proliferates abnormally to a malignant cell population, has created great interest in applying gene therapy techniques to its treatment. Currently, there are 1020 gene therapy studies in clinical trials out of which 675 are cancer related (Table 1)[1]. Many cancer gene therapy strategies focus on transferring genes that either stimulate or increase a specific anti-tumor immune response[2]. These strategies include methods such as the *ex vivo* transfection of T-lymphocytes to tumors and the localized delivery of cytokines that mediate inflammation and an immune response at the tumor site. Gene transfer has also been used to efficiently deliver immune modulating genes into irradiated tumor cells capable of generating a biologically relevant immune response directed toward specific tumor antigens[3].

Many types of cancers evolve as a result of loss or mutation in tumor suppressor genes which act as negative regulatory genes that can overcome the uncontrolled cell proliferation driven by the activated oncogenes or other stimulatory factors. Transduction of these tumor suppressor genes may result in dormancy of the tumor cells or cell death also known as "apoptosis"[4]. An example of such a gene is p53, which has been studied extensively in cancer gene therapy[5]. Viral vectors have also been used for inhibition of the proliferation of tumor cells due to their ability to replicate and lyse tumor cells as part of their life cycle[6]. The inhibition of oncogenes by transduction of antisense oligonucleotides blocking DNA or mRNA is another growing cancer gene therapy approach.

Chemotherapy and gene therapy can be combined via suicide gene therapy where a gene introduced into a tumor converts a systemically non toxic prodrug into a cytotoxic metabolite which not only kills the transduced but also destroys the surrounding cells in a process known as the bystander effect[7]. The most commonly investigated suicide genes are the herpex simplex virus thymidine kinase (HSV-tk) and cytosine deaminase (CD)[3]. HSV-tk gene renders mammalian cells sensitive to the nucleoside analog ganciclovir GCV converted to a phosphorylated compound that inhibits DNA synthesis[8]. The transduction of CD gene results in the intracellular conversion of 5-fluorocytosine into the cytotoxic metabolite 5-fluorouracil, which interferes with DNA and RNA synthesis[9]. Another evolving technique that inhibits tumor growth is the delivery of antiangiogenic genes capable of reducing the tumor vascularization and reducing its proliferation[10, 11].

Barriers to gene transfer

Circumventing the biological barriers to gene delivery is a considerable challenge. Effective cancer gene therapy requires the successful transport of the transgene from the site of administration to the nucleus of the target cells where it becomes available to the transcription machinery or capable of blocking the expression of the defected genes. The barriers facing each vector may vary depending on the route of administration (Figure 1). Biodistribution barriers for systemic gene delivery include the interaction with blood components and nonspecific uptake. Once in blood stream, transgenes are exposed to serum inactivation and degradation by nucleases. Thus, vectors should be capable of protecting DNA and render it inaccessible to these degradative enzymes. Protection could be achieved by encapsulation in protein capsules such as viral vectors, by condensation in polycationic lipid or polymers or by entrapping in controlled release hydrogels. Whereas nonspecific uptake can be reduced by attaching targeting ligands such as antibodies[12] and peptides[13] to the surface of the DNA delivery vector.

At the cell surface, the rate of entry into cells varies with cell type and occurs relatively slowly. After cellular uptake of the gene delivery systems by endocytosis, the endosomal release is another critical barrier which affects the efficiency of gene transfer since most DNA is retained in the endosomes and eventually degraded or inactivated by lysosomal enzymes. A number of strategies have been developed to enhance endosomal release. One involves using fusogenic peptides or lipids to disrupt the endosomal membrane[14]. Another strategy depends on using gene delivery systems with high

buffer capacity known as "proton sponge" presumably able to reduce the acidification of the endosome resulting in swelling and membrane rupture[15].

Following the release from the endosome, the transgene traffics toward the nucleus through the cytoplasm where it can be exposed to degradation by cytosolic nucleases. The transfection efficiency of the DNA may also be reduced by the inability to dissociate from the delivery vector as only free DNA can be transcribed in the nucleus. Whether the nuclear uptake depends on active transport or diffusion or combination thereof, it is not clearly understood. However, the incorporation of short peptide sequences known as "nuclear localization signals" into delivery vectors has shown to enhance DNA delivery to the nucleus via active transport along microtubules[16]. Following nuclear uptake, the transgene is transcribed into messenger RNA (mRNA), which is exported to the cytoplasm and translated to the desired protein.

In addition to those barriers, other biological barriers may challenge the efficiency of cancer gene therapy. For example, crossing the vascular endothelium and migration through the interstitium fluid to reach the tumor cells are also important barriers for the delivery of both systemically and intratumorally administered vectors to solid tumors.

Methods of gene transfer

Naked DNA has been used successfully when locally injected into the tumor[17] or as DNA vaccines[18], however it is highly prone to tissue clearance and inefficient for systematic delivery[19].Vectors for gene delivery can be divided into two major

categories: i) viral vectors such as retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, lentivirus, and vaccinia virus and ii) nonviral methods such as naked DNA, gene gun, liposomes, polymers, peptides and combinations thereof[20].

Viruses have evolved mechanisms of overcoming the most significant biological barriers. Therefore replication-defective viruses with viral coding sequence partially or completely replaced by therapeutic genes have been the focus of many basic research and clinical studies. Although viral vectors are highly efficient in gene delivery they suffer from several drawbacks such as limited DNA loading capacity, toxicity, immunogenicity and potential replication of competent viruses.

The limitations of viral vectors make synthetic vectors an attractive alternative. Non-viral vectors are less toxic and immunogenic and simple to produce on a large scale. Their efficiency, however, is less than that of their viral counterparts. In addition, physical properties such as size and charge play a critical role in their efficiency. In order to increase the transfection efficiency, non-viral delivery systems have been engineered to mimic the viral delivery systems by incorporation of condensing or encapsulation agent to protect DNA from nucleases, targeting ligands to reduce nonspecific uptake, endosomal release agents to avoid lysosomal degradation and nuclear localization signals to enhance nuclear uptake.

Recombinant polymers

Majority of polymers used in drug and gene delivery are synthesized by chemical methods or obtained from natural sources. Chemical synthesis generally produces random copolymers with unspecified monomer sequences and statistical distributions of molecular weights and monomer compositions. This heterogeneity can yield inconsistency in the biological fate and mechanical properties of such polymers. Moreover, chemically synthesized polymers may contain residual organic solvents, which adversely affect DNA and/or viral vectors. Incomplete reactions produce final products with trace amounts of monomer residues which can result in toxicity. Alternatively, naturally-derived polymers, such as collagen matrices used in adenoviral delivery, are limited by batch to batch variability, limited control over crosslinking density and therefore release, and difficulty in introducing new or modified functions (e.g. stimuli-sensitivity, biodegradation, and biorecognition)[21, 22] .

Recombinant DNA technology has allowed the biological synthesis of engineered protein polymers containing repeating blocks of amino acids with precise composition, sequence, and length (reviewed in [23]). Control over the detailed physicochemical properties of the polymers, using recombinant DNA techniques, has important implications in the design of novel biomaterials for controlled gene delivery and other biomedical applications[24]. Precise control over polymer structure using recombinant techniques allows the development of novel gene delivery systems where polymer structure can be correlated with function (i.e., gene transfer)[25]. Table 2 outlines the advantages that recombinant cloning and expression of protein-based polymers provide over naturally occurring and chemically synthesized polymeric biomaterials. Genetically

engineered protein-based polymers include but are not limited to elastin-like polymers, silk-like polymers, silk-elastinlike protein polymers (SELPs), poly (glutamic acid) polymers and alanylglycine polymers[23]. Protein-based polymers can be designed to incorporate a variety of functionalities, including responsiveness to microenvironmental stimuli, controlled biodegradation, and the presentation of informational motifs for cellular and subcellular interactions. Biologically synthesized polymers do not contain toxic monomer residues and solvents and, depending on their structure, can biodegrade to nontoxic amino acids and be eliminated at controlled rates from the body. However, limitations in cloning and expression of cationic polymers in gene delivery must be overcome before the successful evaluation of these systems as systemic gene carriers.

Polymers for systemic gene delivery and controlled release

Most non-viral polymeric gene delivery systems are prepared by condensation of plasmid DNA with cationic vectors to form a dense core of DNA coated with a hydrophilic surface. The transfection efficiency of polymeric gene delivery systems after systemic administration depends on several factors including the physicochemical characteristics of the DNA/polymer complexes such as size and net charge, and presence of targeting, endolytic, and nuclear localization moieties among other factors. In the past decade, many polymers have been investigated for controlled delivery of genes to target cells. These include, but are not limited to, cationic poly (amino acid)s[26], poly vinyl pyrrolidone[27] and chitosans[28]. While the initial stages of polymeric gene delivery primarily involved evaluation of different polymer *types* for gene transfer, recent research is focusing on systematic structure - transfection relationships by variations in the

structure of the related polymers. For example investigators have demonstrated that introduction of histidine[29] or imidazole side chains into poly lysine backbone[30] reduces cytotoxicity and substantially enhances transfection efficiency of the carrier presumably due to the well postulated proton sponge effect[31]. Introduction of cysteine residues has shown to reduce cytotoxicity and increase transfection efficiency in various cancer cell lines in comparison with poly lysines due to the intracellular reduction of disulfide bonds that are formed within the polymeric backbone[32]. Attachment of the fibroblast growth factor (FGF2) to poly lysine as an example of targeting moiety has been shown to enhance transfection efficiency in cells over-expressing FGF2 receptors[33].

Due to inherent limitations in control over structure, current state of the art polymeric gene carriers have serious short comings for correlation of structure with function. For example recently we studied the effect of the feed comonomer composition of a series of closely related random amino acid-based copolymers on the physicochemical properties of their complexes with plasmid DNA and the corresponding protection against degradation by nucleases, cytotoxicity and transfection efficiency at various DNA: polymer ratios[26]. Copolymers of lysine and serine showed protection of DNA against nuclease degradation to a higher extent compared to other copolymers of lysine and alanine or arginine and serine. The presence of arginine residues increased the transfection efficiency of the complexes by 2-3 orders of magnitude, comparable to that of the standard transfection agent Lipofectamine (Figure 2).

Though these studies provided insight into the influence of amino acid composition on transfection, due to the random nature of these copolymers and

heterogeneity in molecular weight it is difficult to establish concise polymer structure / properties relationships. To correlate biological function with polymer structure, it is necessary to have the capability to make constructs with precise structures. Ongoing work in our laboratory involves engineering better defined amino acid based polymers synthesized by recombinant techniques for both localized matrix-mediated as well as systemic administration for cancer gene therapy applications.

Recombinant polymers for matrix-mediated gene delivery

Matrix-mediated gene delivery systems provide several advantages over bolus administration such as the ability to manipulate the release profile to localize the delivery of the transgenes to the target tissue in a sustained and predictable manner, protect DNA from endogenous nucleases when encapsulated in a matrix and delivery of more than one agent at a time for combination therapy. Hydrogel-forming genetically engineered silk-elastinlike protein polymers (SELPs), containing four silk and eight elastin units in the repeat monomer with one elastin unit containing a lysine substitution (namely SELP47K, structure shown in Figure 3A), have been investigated in our laboratory as matrices for controlled gene delivery[34-36]. Results showed that the rate of DNA release from these systems was affected by variation in the ionic strength of the media, hydrogel cure time and polymer concentration[34]. The release of plasmid DNA from the gels fluctuated with changes in the ionic strength of the medium in an "on / off" fashion, which was attributed to the presence of one cationic lysine residue in each monomer unit of the polymer backbone. Plasmid release from recombinant SELP matrices was shown to be both DNA conformation- and size- dependent[35]. The cumulative release of the three

DNA conformations was in the order of linear > supercoiled > open circular, which is assumed to be due to differences in topology of the three conformations and therefore their contact with the hydrogel network. Whereas, the release of a series of plasmids with different sizes showed that the smallest plasmid, pUC18 (2.6 kbp) was released fastest, followed by pRL-CMV (4.08 kbp), pCFB-EGSH-luc (8.5 kbp), and the largest plasmid pFB-ERV (11 kbp), which showed less than 5% release over 28 days[35]. The size-dependent release of plasmid DNA is likely due to the hindered diffusion of larger plasmids through the pores that are formed by physical crosslinking of the hydrogels.

In vitro release studies showed that plasmid DNA remains structurally intact with regard to molecular weight and conformation over 28 days[35]. I*n vivo* delivery evaluated by delivering pRL-CMV, containing the *Renilla* luciferase gene, intratumorally in a murine model of human breast cancer showed that tumor transfection was significantly enhanced for up to 21 days when compared to the delivery of naked pRL-CMV in a hydrogel composition-dependent manner[35].

In addition to delivery of naked DNA, recent research have explored the localized matrix-mediated delivery of viral vectors by natural polymers [21, 22] taking advantage of the greater transfection efficiency provided by the adenoviruses. Compared to natural polymers, recombinant protein-based polymers provide a platform where viral vectors can remain viable for prolonged periods while polymer structure can be tailor-made to achieve desirable release and degradation profiles. The *in vitro* release and bioactivity of adenovirus containing the green fluorescent protein (gfp) gene, as a marker of gene

transfer, for up to 22 days from SELP hydrogels have shown a clear qualitative relationship between the percentage of polymer in the hydrogel and transfection *in vitro*, indicating that the release of adenoviruses can be modulated by changing the composition of the matrix[35].

The physicochemical properties and gene release from hydrogels are not only influenced by polymer concentration as described above, but also by polymer structure and molecular weight. Using genetic engineering techniques we recently biosynthesized a new series of silk-elastinlike polymers, namely SELP 415K (for structure of one prototype see Figure 3B)[36]. These polymers contained eight more elastin units per monomer than SELP 47K (Figure 3A) previously studied for intratumoral gene delivery. The monomer gene segment of the new polymer was designed to encode for 4 silk units and 16 elastin units with one elastin unit containing a lysine residue yielding the monomer repeat of SELP415K. By increasing the length of the elastin block, the distance between silk-like blocks necessary for formation of hydrogen bonds between the polymer chains also increases (Figure 3C). Three SELP 415K analogs with 6, 8 and 10 monomer repeats and precise molecular weights where synthesized. The shear modulus, gel formation, degree of swelling, and sensitivity to environmental stimuli such as pH, temperature and ionic strength of hydrogels made from SELP415K-8mer were compared with SELP47K of similar molecular weight[36]. The new hydrogels had different gelation patterns (Figure 4A) and increased sensitivity of swelling towards changes in temperature (Figure 4B) and ionic strength (Figure 4C) but not pH. SELP 415K containing a reduced number of silk units per polymer chain showed a lower shear

modulus and a delayed formation of non-flowable gels compared to SELP47K. Hydrogels composed of 415K showed an increase in sensitivity towards the length of cure time and exposure to environmental stimuli. Results of swelling studies using 12 wt% 47K and 415K hydrogels disks cured for 4h, 24h and 48h showed that the presence of more silk-like blocks per polymer chain in polymer 47K resulted in an increase in crosslinking density therefore imparting more rigidity to the hydrogel cured for longer durations.

In addition, hydrogels composed of 415K-8mer showed a significant change in their equilibrium swelling ratios in response to changes in temperature (Figure 4B) and ionic strength (Figure 4C). This interesting difference in stimuli sensitivity exhibited by 415K hydrogels, compared to 47K with similar molecular weight, is possibly due to the formation of less rigid, physical crosslinks as caused by a decrease in the ability of the silk-like blocks to impart physical robustness to the hydrogels, and the ability of longer elastin units to self assemble as a function of temperature and ionic strength.

In a more recent work we compared the effect of polymer structure and cure time on DNA release from SELP 47K and 415K hydrogels[37]. Increasing the length of elastin repeating units in the polymer backbone, while maintaining the length of silk repeating units constant, resulted in an increase in the rate of release of plasmid DNA. An increase in cure time resulted in a lower cumulative release for both polymers, however, this effect was more pronounced for SELP 47K where there is a higher degree of interpolymer interaction[37]. The release of DNA from SELP 415K hydrogels showed a

strong dependence on the ionic strength of the medium where more plasmid DNA was released at physiological ionic strength.

Together, these results demonstrate the potential of recombinant techniques to biosynthesize polymers with precise structures for matrix-mediated delivery where it is possible to correlate polymer structure (exact molecular weights and sequences) with its physicochemical properties (e.g., modulus, gel formation, swelling, and sensitivity to environmental stimuli) and gene release.

Recombinant polymers for systemic gene delivery

While matrix-mediated gene delivery provides distinct advantages over systemic administration as described above, its use for the treatment of solid tumors is generally limited to intratumoral injection. As a first step towards the development of recombinant polymers for systemic cancer gene therapy, recently we have genetically engineered and characterized a gene carrier with the following structure $(KHKHKHKHKK)_6$-FGF2 for systemic gene delivery[38]. The proposed carrier contains, at precise locations on the polymeric backbone, lysine (K) residues to condense plasmid DNA, a targeting ligand (basic fibroblast growth factor, FGF2) for biorecognition by cancer cells expressing FGFR (Fibroblast Growth Factor Receptor), and histidine (H) residues to allow escape from the endosomes and DNA release. Though polymeric gene carriers with one or more of similar functional moieties have been reported in the literature[33, 39], they have been synthesized by traditional chemical techniques. In this approach we have combined

biosynthetic (at the gene construct level) motifs necessary for successful gene transfer in a polymer-protein fusion with exquisite control over sequence and length.

A key component of the cloning and expression of such carrier is the cloning of the lysine-histidine monomers and the corresponding multimerization of the repeating units. Cloning of repeating residues is challenging due to increased probability of recombination and / or deletion during the cloning process. In addition the head and tail sequences can introduce extra and unnecessary base pairs encoding amino acids other than the ones intended. The general strategy for the biosynthesis of this first recombinant gene carrier for systemic gene delivery is described in Figure 5. The strategy was to first stably clone and obtain enough copies of the gene monomer segments encoding for lysine and histidine unit $(KHKHKHKHKK)_1$, abbreviated hereafter as $(KH)_1$, for further multimerization. The oligonucleotides encoding lysine-histidine $(KH)_1$ monomers were designed to maximize the use of preferred codons in *E. coli*, while minimizing the codon repetition of the monomer gene. Restriction sites used for cloning into the cloning vector (pZero-2) (Invitrogen, Carlsbad, CA) and the expression vector (*pAAG*) were also included. Oligonucleotides encoding the monomer were first annealed and digested with *Bam*HI and *Eco*RI (New England Biolabs, Beverly, MA) restriction enzymes. Simultaneously, the pZero-2 cloning vector was digested with the same enzymes. After removal of the enzymes, the monomer DNA and vector were ligated and transformed into TOP10 cells. The monomer gene segment encoding for 10 repeats of lysine and histidine (total 20 amino acids) was stably cloned. The number of histidine residues was 40% of the total lysine-histidine residues. This is based on previous studies demonstrating that a

chemically synthesized poly lysine polymer, containing 38 ± 5% of the ε-amino groups of lysine substituted with histidyl residues, mediated transfection several orders of magnitude greater than polylysine, polylysine + chloroquine, or polylysine + E5CA (an endosomolytic peptide)[29].

In the next step the gene monomers were self-ligated using seamless cloning technique[40] to produce multimer gene segments or concatemers. A custom-designed vector was made for cloning of the KH concatemers. This vector was named *pAAG*, because of its engineered 5' three-base AAG overhang. The basis for this vector was the pET-19b (Novagen, Madison, WI) expression vector, which contains a histidine tag at the N-terminal of the cloning site, followed by an enterokinase cleavage site. After preparation of the purified concatemer and vector, the two were ligated with T4 DNA ligase. The ligation mixture was used to transform *E. coli* TOP10. Colonies were screened by PCR colony screening, to determine the approximate size of the insert. The size and sequence of the insert was verified by triple DNA sequencing. Results showed stable cloning of lysine-histidine repeating units.

In parallel, the FGF2 gene was amplified by PCR from a plasmid to produce X-FGF2 (Figure 5). X-FGF2 was the FGF2 gene which had proper restriction sites for fusion with $(KH)_6$ gene. X-FGF2 gene was first digested with *NdeI* and *HindIII* and cloned into pET21b to make pET21b-X-FGF2. $(KH)_6$ gene which corresponds to 60 lysine-histidine residues was amplified from the p*AAG vector* by PCR. $(KH)_6$ gene and pET21b-X-FGF2 were both digested with *NdeI* and *EcoR I* and ligated using Quick T4

DNA Ligase. The cloned vector was sequenced and insertion of both $(KH)_6$ and FGF2 gene into pET21b was confirmed. pET21b-$(KH)_6$-FGF2 vector was transformed into protease deficient BL21(DE3) (*lon*$^-$, *ompT*$^-$) host (Novagen Madison, WI) and expressed. The copolymer expression was identified by SDS-polyacrylamide gel electrophoresis and western blot analysis. SDS-PAGE results estimated the molecular weight of the protein to be approximately 30,000 daltons while western blot analysis confirmed the presence of 6 sequential histidine residues in the structure of the expressed protein. Theoretically, the expressed protein has a molecular weight of approximately 27,313 daltons. The exact molecular weight of the protein was determined by MALDI-TOF to be 27,402 which was close to the theoretical value of 27,313 daltons.

The methods described in this review demonstrate the successful cloning and expression of the first prototype recombinant gene carrier for systemic gene delivery. Currently, our laboratory is evaluating the mitogenic activity, toxicity and *in vitro* transfection efficiency of $(KH)_6$-FGF2. The next logical steps in this research are to systematically vary carrier structure using recombinant techniques, and correlate this structure with gene transfer in vitro and in vivo. In the long-term this can lead to the development of clinically acceptable targetable carriers for cancer gene therapy applications with appropriate transfection efficiency profiles and reduced toxicity.

The use of recombinant polymers for controlled gene delivery is in its infancy. The full potential of genetically engineered polymers for this purpose will be realized by further systematic polymer structure / delivery relationships. As we learn more about

structural motifs responsible for protection of the genetic material from degradation, targeting to the desired site of action, gene release, and subcellular trafficking, novel delivery systems can be designed to overcome these barriers. Alterations in gene carrier structure can be based on the mechanistic understanding of the steps of gene transfer process. The foreseeable challenges are the stable cloning and expression of cationic polymers with repetitive amino acid sequences, identifying methods to overcome or reduce immunogenicity of such constructs, as well as reducing the initial high cost of the production of such polymers.

Acknowledgements:

Financial support was made possible by grants from the NIH (R01CA107621-01), DOD (DAMD17-03-0237 and DMAD17-03-1-0534) and the National Cancer Center Postdoctoral Fellowship (AH).

Table 1. Diseases addressed by gene therapy clinical trials*

Diseases	Protocols	
	Number	**%**
Cancer	675	66
Monogenic diseases	93	9.4
Infectious diseases	68	6.6
Vascular diseases	85	8.1
Other diseases	31	2.9
Gene Marking	52	5.3
Healthy Volunteers	16	1.2
Total	1020	100

*Adapted from reference [1] with permission.

Table 2. Comparison between chemical synthesis and genetic engineering of polymers

Chemical Synthesis	Genetic Engineering
• Polydisperse	• Theoretically monodisperse
• Monomer sequence generally not well-defined	• Well-defined monomer sequence
• Difficult to achieve stereoregularity	• Polymers obtained are stereoregular
• Difficult to incorporate motifs from nature	• Ability to incorporate motifs from nature
• Initial production generally requires lower costs	• Expensive initial design and production
• Procedure can be standardized	• Depends on biological systems that are difficult to standardize

Figure 1: Barriers to systemic and local gene delivery: (1) complex formation and characterization, (2) transport in blood circulation, (3) uptake/entry into the cell, (4) release from endosome, (5) dissociation from synthetic vector, (6) transit from cytoplasm to nucleus, (7) uptake/entry into nucleus and (8) transgene expression.

Figure 2: Transfection of Cos-7 cells after incubation with plasmid DNA / polymer complexes at different DNA: polymer wt/wt ratios: (◆) poly [(Lys, Ala) 1:1], (✱) poly [(Lys, Ala) 2:1], (▲) poly [(Lys, Ala)] 3:1, (✕) poly [(Lys, Ser) 3:1], (■) poly [(Arg, Ser) 3:1], (●) Poly-L-Lys. (Values are reported as average of n = 9 ± SD where n represents the number of times each complex was made and transfection efficiency determined). From reference [26] with permission.

Figure 3. The amino acid sequence of: **A.** SELP47K (molecular weight 69,814 Da); **B.** SELP415K (molecular weight 71,500 Da); **C.** Schematic of SELP47K and SELP415K showing hydrogen bonds between silk-like blocks as points of crosslinking between polymer chains. Elastin like units containing a lysine residue are designated with positive charge. Adapted from reference [36] with permission.

Figure 4. A. Effect of cure time on the degree of swelling (q=wt. of wet gel/wt. of dry gel) as a function of polymer structure; **B.** Effect of temperature in 1X PBS (pH 7.4, μ = 0.16); **C.** Effect of ionic strength at 37 °C in phosphate buffer solution (pH 7.4) (■) SELP415K, (□) SELP47K. Bars and symbols represent mean value ± standard deviation (n = 3). Adapted from reference [36] with permission.

Figure 5: Overview of the cloning strategy used to prepare $(KH)_6$-FGF2 gene. For abbreviations see text.

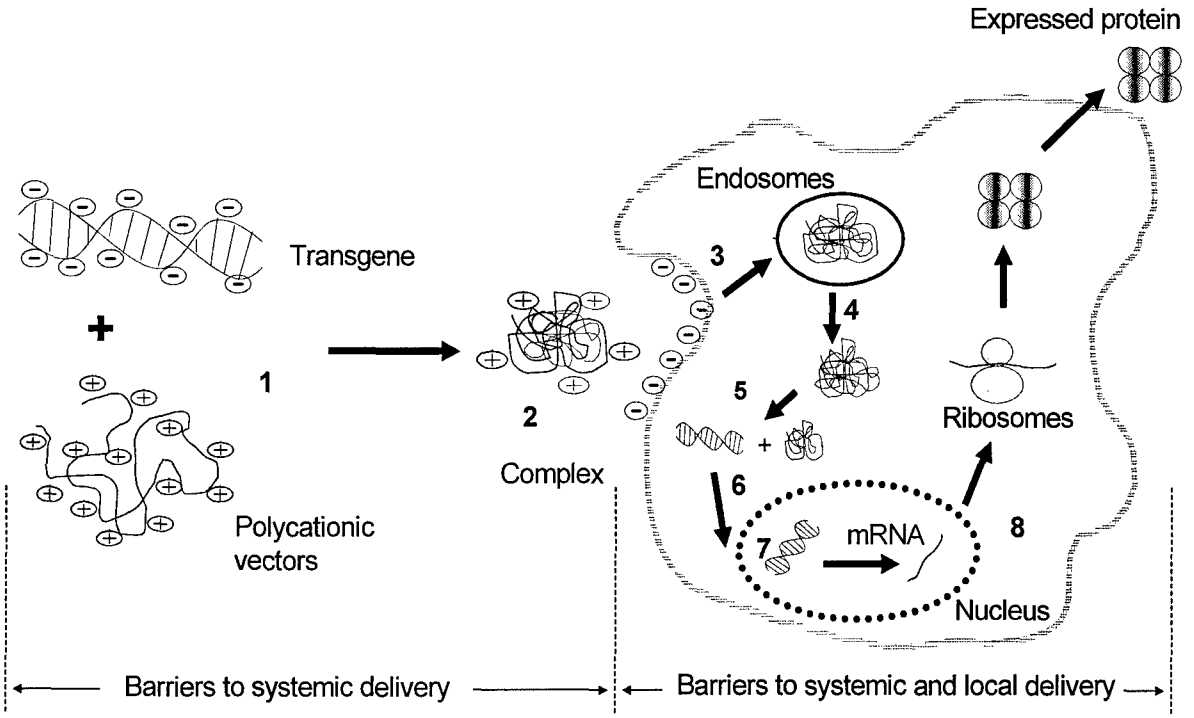

Expressed protein
Endosomes
Transgene
3
4
1
5
2
Ribosomes
Complex
6
Polycationic
vectors
7
mRNA
8
Nucleus
Barriers to systemic delivery
Barriers to systemic and local delivery

Figure 1

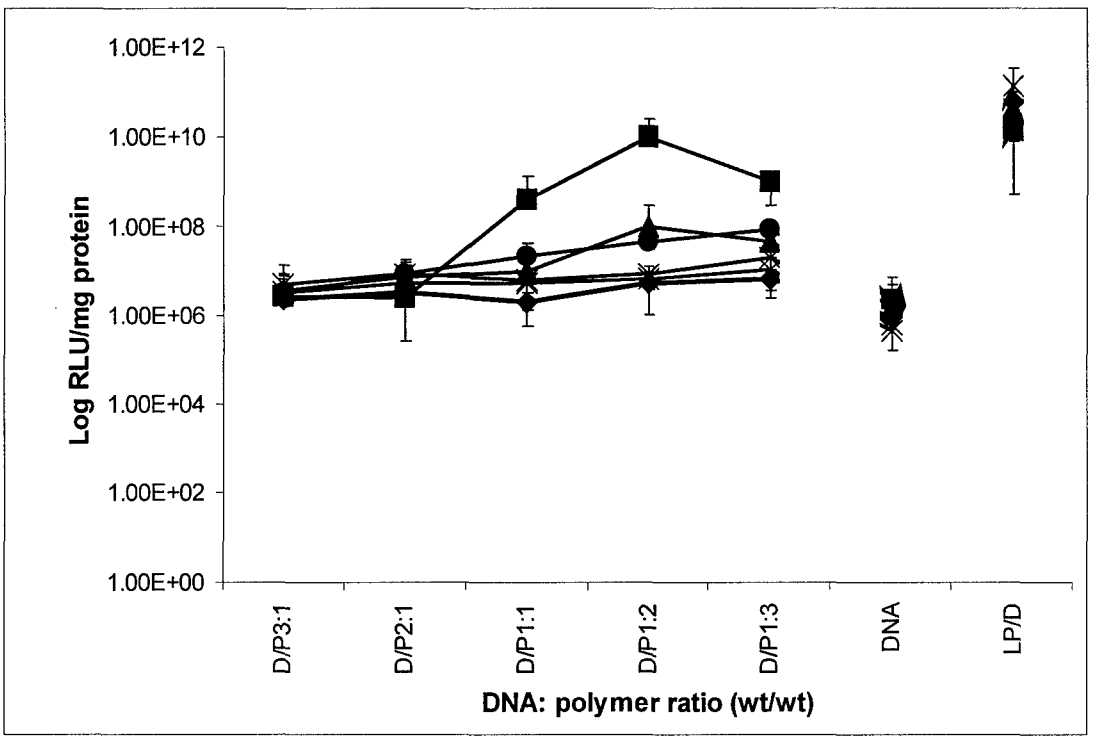

1.00E+12
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
Log RLU/mg protein
D/P3:1
D/P2:1
D/P1:1
D/P1:2
D/P1:3
DNA
LP/D
DNA: polymer ratio (wt/wt)

Figure 2

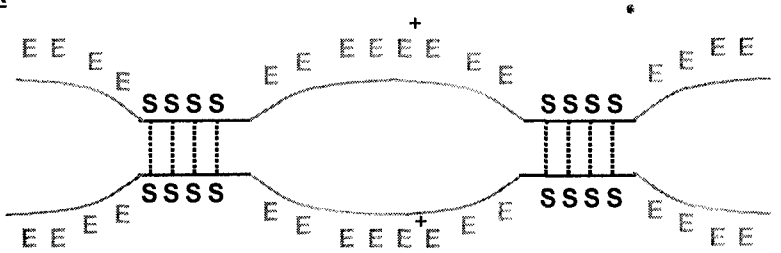
A. ***SELP 47K***

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSG**AGS [(GVGVP)$_4$ GKGVP (GVGVP)$_3$ (GAGAGS)$_4$]$_{12}$ (GVGVP)$_4$ GKGVP (GVGVP)$_3$ (GAGAGS)$_2$ GAG**AMDPGRYQDLRSHHHHHH

B. ***SELP 415K***

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSG**AGS [(GVGVP)$_4$ GKGVP (GVGVP)$_{11}$ (GAGAGS)$_4$]$_7$ (GVGVP)$_4$ GKGVP (GVGVP)$_{11}$ (GAGAGS)$_2$ GAG**AMDPGRYQDLRSHHHHHH

C. ***SELP 47K***



SELP 415K

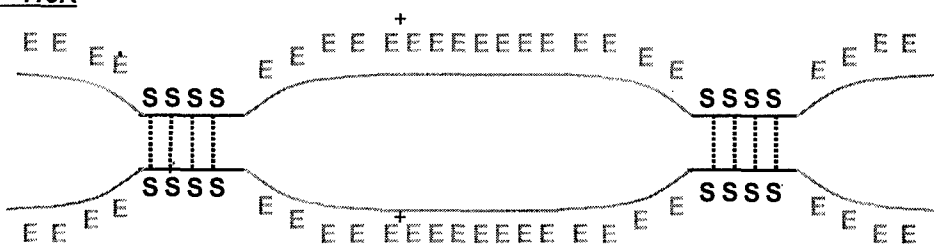


Figure 3

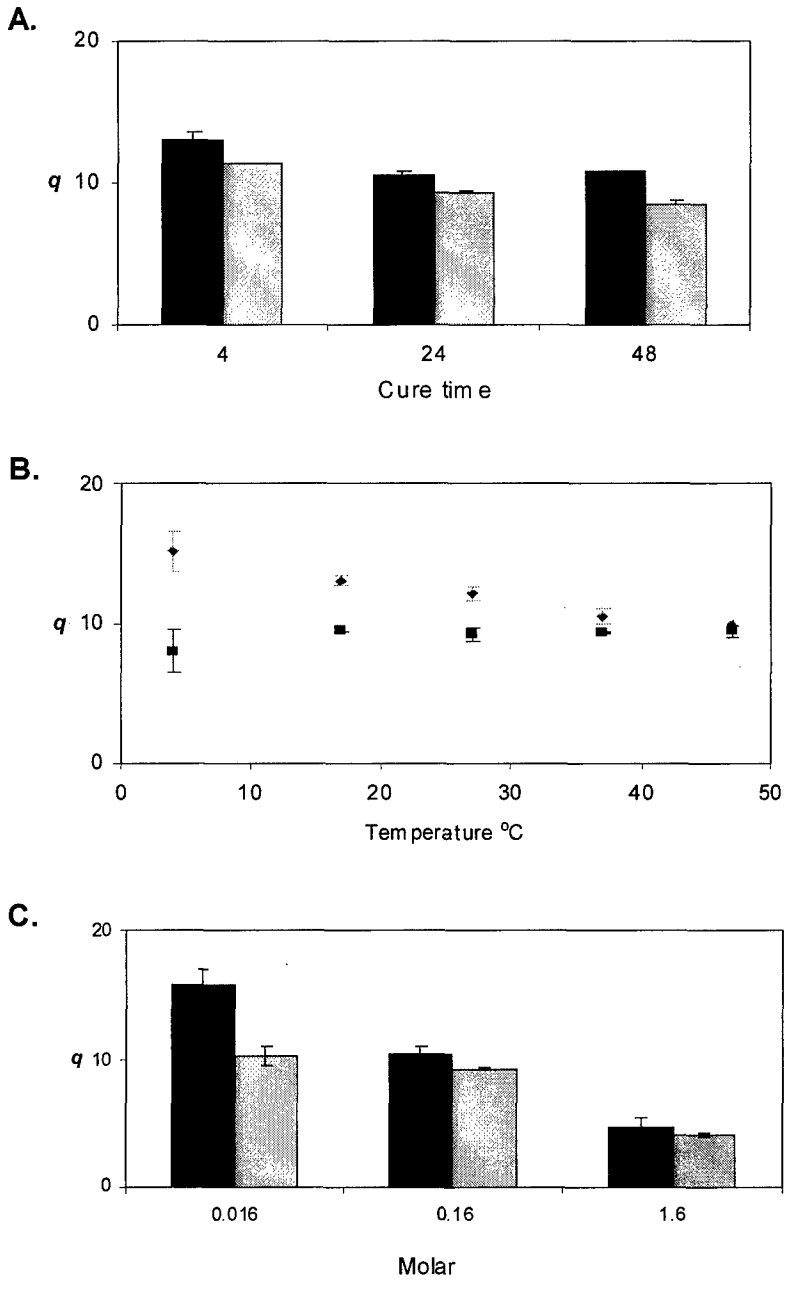

A.
20
q 10
0
4
24
48
Cure time
B.
20
q 10
0
0
10
20
30
40
50
Temperature °C
C.
20
q 10
0
0.016
0.16
1.6
Molar

Figure 4

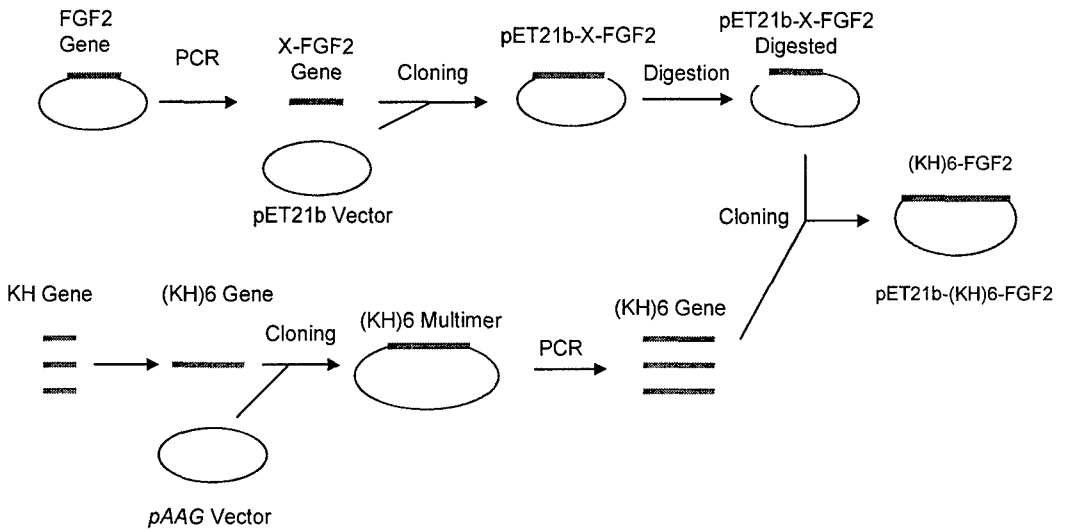

FGF2
Gene
PCR
X-FGF2
Gene
Cloning
pET21b Vector
pET21b-X-FGF2
Digestion
pET21b-X-FGF2
Digested
Cloning
(KH)6-FGF2
pET21b-(KH)6-FGF2
KH Gene
(KH)6 Gene
Cloning
(KH)6 Multimer
pAAG Vector
PCR
(KH)6 Gene

Figure 5

References:

1. Gene therapy clinical trials, J Gene Med, Accessed May 2005, http://www.wiley.co.uk/genetherapy/clinical.
2. D. W. Kowalczyk, P. J. Wysocki, and A. Mackiewicz. Cancer immunotherapy using cells modified with cytokine genes. *Acta Biochim Pol* **50**: 613-624 (2003).
3. B. W. O' Malley, and M. E. Couch. In A. Roland (ed), *Advanced Gene Delivery*, Hardwood Academic Publishers, Amsterdam, 1999, pp. 281-310.
4. L. R. Jiao,and N. A. Habib. Experimental study of large-volume microwave ablation in the liver. *Br J Surg* **89**: 1003-1007 (2002).
5. E. R. Sauter, R. Takemoto, S. Litwin, and M. Herlyn. p53 alone or in combination with antisense cyclin D1 induces apoptosis and reduces tumor size in human melanoma. *Cancer Gene Ther* **9**: 807-812 (2002).
6. C. Heise, A. Sampson-Johannes, A. Williams, F. McCormick, D. D. Von Hoff, and D. H. Kirn. ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med* **3**: 639-645 (1997).
7. D. Kirn. Virotherapy for cancer: current status, hurdles, and future directions. *Cancer Gene Ther* **9**: 959-960 (2002).
8. F. L. Mooltenand J. M. Wells. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. *J Natl Cancer Inst* **82**: 297-300 (1990).
9. L. A. Martin,and Lemoine N.R. Direct cell killing by suicide genes. *Cancer Metastasis Rev* **15**: 301-316 (1996).
10. P. Lin, J. A. Buxton, A. Acheson, C. Radziejewski, P. C. Maisonpierre, G. D. Yancopoulos, K. M. Channon, L. P. Hale, M. W. Dewhirst, S. E. George, and K. G. Peters. Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tier2. *Proc Natl Acad Sci USA* **95**: 8829-8834 (1998).
11. C. K. Goldman, R. L. Kendall, G. Cabrera, L. Soroceanu, Y. Heike, G. Y. Gillespie, G. P. Siegal, X. Mao, A. J. Bett, W. R. Huckle, K. A. Thomas, and D. T. Curiel. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc Natl Acad Sci U S A* **95**: 8795-8800 (1998).
12. C. H. Lee, M. Hsiao, Y. L. Tseng, and F. H. Chang. Enhanced gene delivery to HER-2-overexpressing breast cancer cells by modified immunolipoplexes conjugated with the anti-HER-2 antibody. *J Biomed Sci* **10**: 337-344 (2003).
13. M. Shadidi, and M. Sioud. Identification of novel carrier peptides for the specific delivery of therapeutics into cancer cells. *Faseb J* **17**: 256-258 (2003).
14. E. Mastrobattista, G. A. Koning, L. van Bloois, A. C. Filipe, W. Jiskoot, and G. Storm. Functional characterization of an endosome-disruptive peptide and its application in cytosolic delivery of immunoliposome-entrapped proteins. *J Biol Chem* **277**: 27135-27143 (2002).
15. Y. W. Cho, J. D. Kim, and K. Park. Polycation gene delivery systems: escape from endosomes to cytosol. *J Pharm Pharmacol* **55**: 721-734 (2003).

16. M. A. Zanta, P. Belguise-Valladier, and J. P. Behr. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci USA* **96**: 91-96 (1999).
17. F. Shi, A. L. Rakhmilevich, C. P. Heise, K. Oshikawa, P. M. Sondel, N. S. Yang, and D. M. Mahvi. Intratumoral injection of interleukin-12 plasmid DNA, either naked or in complex with cationic lipid, results in similar tumor regression in a murine model. *Mol Cancer Ther* **1**: 949-957 (2002).
18. P. Hanke, M. Serwe, F. Dombrowski, T. Sauerbruch, and W. H. Caselmann. DNA vaccination with AFP-encoding plasmid DNA prevents growth of subcutaneous AFP-expressing tumors and does not interfere with liver regeneration in mice. *Cancer Gene Ther* **9**: (2002).
19. K. Kawabata, Y. Takakura, and M. Hashida. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm Res* **12**: 825-830 (1995).
20. A. El Aneed. An overview of current delivery systems in cancer gene therapy. *J Controlled Release* **94**: 1-14 (2004).
21. L. A. Chandler, J. Doukas, A. M. Gonzalez, D. K. Hoganson, D. L. Gu, C. Ma, M. Nesbit, T. M. Crombleholme, M. Herlyn, B. A. Sosnowski, and G. F. Pierce. FGF2-Targeted adenovirus encoding platelet-derived growth factor-B enhances de novo tissue formation. *Mol Ther* **2**: 153-160 (2000).
22. J. Doukas, L. A. Chandler, A. M. Gonzalez, D. Gu, D. K. Hoganson, C. Ma, T. Nguyen, M. A. Printz, M. Nesbit, M. Herlyn, T. M. Crombleholme, S. L. Aukerman, B. A. Sosnowski, and G. F. Pierce. Matrix immobilization enhances the tissue repair activity of growth factor gene therapy vectors. *Hum Gene Ther* **12**: 783-798 (2001).
23. M. Haider, Z. Megeed, and H. Ghandehari. Genetically engineered polymers: status and prospects for controlled release. *J Controlled Release* **95**: 1-26 (2004).
24. J. Cappello, and H. Ghandehari, (eds.). Engineered Protein Polymers for Drug Delivery and Biomedical Applications. *Adv Drug Del Rev* **54**: 1053-1159 (2002).
25. Z. Megeed, and H. Ghandehari. Genetically Engineered Protein-Based Polymers: Potential in Gene Delivery. In Amiji M. (ed), *Polymeric Gene Delivery: Principles and Applications*, CRC Press, Boca Raton, FL, USA, 2005, pp. 489-507.
26. M. Haider, and H. Ghandehari. Influence of poly (amino acid) composition on complexation with plasmid DNA and transfection efficiency. *J Bioact Compat Pol* **11**: 93-111 (2003).
27. R. J. Mumper, J. G. Duguid, K. Anwer, M. K. Barron, H. Nitta, and A. P. Rolland. Polyvinyl derivatives as novel interactive polymers for controlled gene delivery to muscle. *Pharm Res* **13**: 701-709 (1996).
28. K. Roy, H. Q. Mao, S. K. Huang, and K. W. Leong. Oral gene delivery with chitosan--DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat Med* **5**: 387-391 (1999).
29. P. Midoux, and M. Monsigny. Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjugate Chem* **10**: 406-411 (1999).

30. D. Putnam, C. A. Gentry, D. W. Pack, and R. Langer. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc Natl Acad Sci USA* **98**: 1200-1205 (2001).
31. J. P. Behr. The proton sponge: A trick to enter cells the viruses did not exploit. *Chimia* **51**: 34-36 (1997).
32. M. L. Read, K. H. Bremner, D. Oupicky, N. K. Green, P. F. Searle, and L. W. Seymour. Vectors based on reducible polycations facilitate intracellular release of nucleic acids. *J Gene Med* **5**: 232-245 (2003).
33. B. A. Sosnowski, A. M. Gonzalez, L. Chandler, Y. J. Buechler, G. F. Pierce, and A. Baird. Targeting DNA to cells with basic fibroblast growth factor (FGF2). *J Biol Chem* **271**: 33647-53 (1996).
34. Z. E. Megeed, J. Cappello, and H. Ghandehari. Controlled release of plasmid DNA from a genetically engineered silk-elastinlike hydrogel. *Pharm Res* **19**: 954-959 (2002).
35. Z. Megeed, M. Haider, D. Li, B. W. O'Malley Jr., J. Cappello, and H. Ghandehari. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. *J Controlled Release* **94**: 433-445 (2004).
36. M. Haider, V. Leung, F. Ferrari, J. Crissman, J. Cappello, and H. Ghandehari. Molecular Engineering of Silk-elastinlike Polymers for Matrix-Mediated Gene Delivery: Biosynthesis and Characterization. *Mol Pharm* **2**: 139-150 (2005).
37. V. Moolchandani, M. Haider, and H. Ghandehari. In Vitro Release of Plasmid DNA from Structurally Related Silk-Elastinlike Hydrogels. *32nd Annual Meeting & Exposition of the Controlled Release Society* (2005).
38. A. Hatefi,and H. Ghandehari. Design and Biosynthesis of a Genetically Engineered Non-Viral Vector for Breast Cancer Gene Therapy, *Proceedings of the 32nd Annual Meeting & Exposition of the Controlled Release Society*, Miami, Florida, USA, 2005.
39. A. Andersson, J. Capala, and J. Carlsson. Effects of EGF-dextran-tyrosine-131I conjugates on the clonogenic survival of cultured glioma cells. *J Neurooncol* **14**: 213-23 (1992).
40. N. L. Goeden-Wood, V. P. Conticello, S. J. Muller, and J. D. Keasling. Improved assembly of multimeric genes for the biosynthetic production of protein polymers. *Biomacromolecules* **3**: 874-879 (2002).